OPIC
CIPO
(21) (A1) 2,180,786
(22) 1996/07/09
(43) 1998/01/03

(51) Int.Cl.[6] C12N 15/53; C12N 5/10; A01H 5/00

(19)(CA) APPLICATION FOR CANADIAN PATENT (12)

(54) Plants Containing the gdhA Gene and Methods of Use Thereof

(72) Lightfoot, David - U.S.A. ;
Long, Lynn - U.S.A. ;
Lightfoot, Maria Vidal - U.S.A. ;

(71) Board of Trustees of Southern Illinois University - ;

(30) (US) 60/021,058 1996/07/02

(57) 28 Claims

ABSTRACT

Plants transformed with a gdhA gene and also with a gene used as a selectable marker provide a dual gene herbicide resistant and tolerance package. The transgenic plants and their progeny exhibit an expression cassette having transcription initiation and transcription termination regions functional in the plant cells, and a DNA sequence encoding the GDH enzyme. The expression cassette imparts a detectable level of herbicide resistance to the phosphinothricin class of herbicides. Transformed cells may further include a marker gene, such as the phosphinothricin acetyl transferase gene and/or the Bar gene. Plants having this expression cassette can be grown in an environment including a phosphinothricin class herbicide to control undesirable vegetation without significantly affecting crop growth.

PLANTS CONTAINING THE *gdhA* GENE AND METHODS OF USE THEREOF

FIELD OF THE INVENTION

The present invention relates to plants transformed with the *gdhA* gene. More specifically, the present invention relates to a gene which can be used as a selectable marker in transformation. Additionally, the present invention relates to a dual gene herbicide resistance and tolerance package that includes the phosphinothricin acetyl transferase (PAT) gene and/or the Bar gene in combination with the *gdhA* gene.

BACKGROUND OF THE INVENTION

Plants utilize nitrogen to form organic compounds. Ammonia and ammonium ions do not accumulate in plants cells but instead are rapidly assimilated. Ammonium assimilates through two possible pathways. The first pathway produces glutamate and is catalyzed by glutamate dehydrogenase (GDH), which is found in chloroplasts and mitochondria.

The second pathway for assimilation of ammonia invclves a reaction with glutamate to form its amide, glutamine. This reaction is catalyzed by glutamine synthetase (GS) and requires energy in the form of ATP. Glutamine is then catalyzed by glutamate synthase (GOGAT) to form glutamate. GS appears in chloroplasts and cytosol in leaves and roots, whereas, GOGAT is in leaf chloroplasts and plastids in roots.

Although both pathways result in glutamate, the second pathway appears more important in ammonium assimilation in plants. Glutamate dehydrogenase, the enzyme of the first pathway, has a high Km value. This value which is the concentration of ammonia where half of the enzyme maximum operation rate is within levels which are toxic for plant

cells. In contrast, the GS Km value is much lower. Additionally, radioactive labeling of NO_3 or NH_4 show labeled nitrogen in the amide group of glutamine first.

Although GS has a high affinity for ammonia and GDH has a lower affinity, GS has low specific activity per enzyme molecule and GDH has high specific activity per molecule.

Ammonium assimilation pathways of plants and microorganizm; although maybe not fully understood; have been known. In October of 1980, the ICI Agricultural Division published in Nature, Volume 287, page 396 an article on improved conversion of methanol to single cell protein by *Methylophilus methylotropus*.

The researchers cloned the glutamine dehydrogenase gene of *Escherichia coli* (*E. coli*) into a mutant of *Methylophilus methylotropus* organism that lacks GOGAT. The paper explained that the GDH pathway should result in the organism consuming less energy. The researchers speculate that potential industrial or agricultural savings could be made by identification of features that incur "energy penalty" and this is an exciting area for recombinant DNA. This organism to organism transfer of the *E. coli* GDH gene should substantially decrease in enzyme activity thus a plasmid with a high copy number was used.

In 1988, the expression of *E. coli* glutamate dehydrogenase in cyanobacterium was reported in Plant Molecular Biology, Volume II, pages 335-344. Cyanobacterium that lacked glutamate dehydrogenase were transformed with the *gdhA* gene of *E. coli* and levels of NADP-specific glutamate dehydrogenase activity resulted in the transformed microorganism. The authors speculate that it would be interesting to investigate the engineering of glutamate

dehydrogenase activity to higher plants and to study in detail the possible roles for glutamate dehydrogenase activity in ammonium detoxification.

Although there was some speculation on nitrogen assimilation genes in higher plants, in a paper on nitrogen assimilatory genes in The Genetic Manipulation of Plants and its Application to Agriculture, at page 109, the authors state that it would be tempting to suggest that crop plants might show increased metabolic efficiency if ammonium assimilation was channeled through glutamate dehydrogenase. But the authors clearly list the number of technological barriers to this. There remained a number of barriers to this research including the potential negative consequences of uncontrolled expression in the plant. The authors reluctantly conclude "perhaps" there may be some benefit in replacing glutamate synthase, with ammonium - utilizing alternatives.

In Molecular and General Genetics in 1993 in volume 236, pages 315-325, the modulation of glutamine synthetase gene expression in tobacco was reported. An alfalfa gene was placed in the tobacco plant cells in the sense and antisense position. Partial inhibitation in the antisense position was seen without a true homologous gene.

In 1994, it was reported that increasing the activity of plant nitrogen metabolism enzymes may alter plant growth, development and composition. Increased yield and protein content as well as reduced levels of nitrogen in agricultural runoff water and food may result. Plant nitrogen metabolism has been altered by transformation with a highly active assimilatory bacterial glutamate dehydrogenase gene, plant glutamate dehydrogenase is less active in ammonium gene has been altered by PCR and PCR strand overlap exchange to modify

coding region and allow high levels of expression in plant cells. The 5' non-coding region has been altered to increase translation and permit protein targeting to either cytosol or chloroplasts. The 3' non-coding region has been altered to stabilize the mRNA and ensure appropriate polyadenylation of the mRNA. Certain codons likely to inhibit expression to high levels in plant cells have been altered. The effects of the various sequence substitutions on gene expression in plant cells compared to the unmodified gene will be reported. This abstract is reporting on speculation of the researchers as the abstract clearly reference what may happen or codons that are likely to inhibit. The abstract appears to provide a guess as to what might happen, not something that has been done.

Although researchers speculated that the *gdhA* gene may be useful in higher plants, the drawbacks and possible disruption of the photosynthesis pathway lead researchers to the belief that the potential use was probably not possible due to technical barriers. Even the inventor was only speculating on the potential of the *gdhA* gene to avoid ammonia toxification.

There remains a need to transform cereals to determine if the *gdhA* gene would have any effect on the plant in either nontoxifying levels or toxic levels of ammonia. The usefulness of the gene as a tolerance mechanism for certain herbicides was not proven prior to this. The combination of this *gdhA* gene with other selectable markers to increase plant resistance to herbicide damage was heretofore undiscovered. The ability of a plant to increase dry weight due to increased nitrogen uptake in even nontoxic levels of ammonia was not realized or considered until the present invention.

question remains if th supply of nitrogen is at a high level can the composition of prot ins, sugars, starch, cellulose, lipids and oils be modified by the addition of the *gdhA* gen .

The present invention clearly indicates that the protein content in seeds and leaves is altered. Although the *gdhA* gene may have had some suggested potential to assimilat additional nitrogen in highly toxic nitrogen conditions, th *gdhA* genes result GDH enzyme has a weaker ammonium affinity than the ATP specific GS. At lower ammonium concentrations assimilation by GDH was expected to be limited due to its lower ammonium affinity and the reversibility of its reaction.

Thus, it was surprising and unexpected that the *gdhA* gene when in a plant produced measurable changes in the number of leaves and protein content of the leaves and the seeds, the dry weight of the plant even in soils having normal ammonium levels. At these levels, the expectation would be that the GS / GOGAT cycle would be the active cycle.

SUMMARY OF THE INVENTION

An object of this invention is to provide transformed plants containing the *gdhA* gene that evidences increased plant biomass.

Another object of this invention is to provide transformed plants that increases leaf size.

Still another object of this invention is to provide transformed corn plants that are resistant to PPT which includes phosphinothricin and glufosinate herbicides and the acid and salt derivatives and may extend to organophosphorus amino acid herbicides such as Bialaphos.

Yet a different object of this invention is to provide a corn plant with dual gene resistance to PPT in the GS and the GDH pathways.

Furthermore, an object of the present invention is to provide altered plant growth and yield in seed crops including sunflower, corn, soybeans and canola (brassica).

Additionally, the object of the present invention is to provide a *gdhA* transformed corn plant that contains a gene that alters the composition of the makeup of the corn seed.

Broadly, then the present invention includes a method of improving crop growth by applying to a field containing a crop, which are phosphinothricin resistant due to having an expressable transgene encoding for phosphinothricin resistant glutamate dehygrodenase enzyme, a sufficient amount of a phosphinothricin class herbicide to control undesirable vegetation without significantly affecting crop growth.

This method includes a gene which is mutagenized, and a gene which is a modified bacterial gene. The gene can contain the Kozac consensus sequence in a particular embodiment. This method, of course, can include instances where the phosphinothricin class herbicide is combined with a second herbicide and then applied to the transformed crop.

The method includes transformed crops which are selected from the group consisting of corn, cotton, brassica, soybeans, wheat or rice. Some of these crops are naturally resistant and the addition of the *gdhA* allows additional heartiness during herbicide application.

This invention is not just about the method described about. This invention also includes within its broad scope.

Transgenic plant cells and progeny having expression cassettes with a transcription initiation region functional in the plant cells, a DNA sequence that encodes for the GDH enzyme in said plant cells, and a transcription termination region functional in the plant cells. The expression cassette then imparts to the plant a detectable level of herbicide resistance to the phosphinothricin class of herbicides.

In the cells at least one of the transcription region or the termination region is not naturally associated with the *gdhA* sequence. The invention encompass these cells wherein the sequence is from a bacterial gene preferably from *E. coli*. In some embodiments these cells, including a sequence from the bacterial gene, are modified to enhance expression in plant cells. The cells, plants and progeny include a DNA sequence that encodes the amino acid sequence shown in Figure 3.

To enhance amino acid production, the cells can include chloroplast transient peptide under sequences adapted to target the chloroplasts. In other embodiments, cells have a transcription initiation region which constitutive in action or can be organ or tissue specific.

The present invention includes cell culture of cells that contain a marker gene that is capable of growth in a culture medium which includes a herbicide which is in the phosphinothricin class. Additionally, the present invention includes a cell culture of cells having a gene resistant to the PPT and a marker gene that is capable of growth in a culture medium which includes a herbicide which is not a phosphinothricin class herbicide. The herbicide includes bialaphos and Ignite™.

A transgenic plant originally formed from nontransgenic plants and progeny thereof which contains an expression cassette having a transcription initiation region functional in the plant cell, a genetically engineered DNA sequence that is capable of encoding for the GDH enzyme in the plant cells wherein the plant evidences detectable alteration in GDH activity when compared to the nontransgenic plants like that from which the transgenic plant was formed. The alteration in GDH activity could be increased activity or decreased activity. The transgenic plant can be a dicot or a monocot. Of particular interest are transgenic *Zea mays* plants. Alternatively, the transgenic plant can be selected from a group consisting of brassica, cotton, soybeans, and tobacco. The change in the nitrogen assimilation pathway allows other parts of the plant to be altered.

Thus, a transgenic plant that plant forms seeds and has genetically engineered DNA sequences that alters the oil content of the seed of the plant and evidences altered GDH activity when compared to a transgenic plant containing only the oil altering DNA sequence.

The invention covers a transformed corn plant containing a bacterial glutamate dehydrogenase gene. Additionally, this plant can contain a second gene that was introduced into the plant or its ancestors by genetic engineering that is resistant to PPT.

The invention broadly covers a recombinant plasmid characteristic in that the recombinant plasmid contains a constitutive promoter, a chloroplast transit peptide and the bacterial *gdhA* gene and a transcriptional termination region. A biologically pure culture of a bacterium characterized in that the bacterium is transformed with the recombinant plasmid.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows the DNA sequence of the *gdhA* of *E. coli*.

FIG. 2 shows the forward primer at 5' and the reverse primer at 3' of the non-coding region of the *gdhA* gene. SacI and XbaI restriction enzyme sites are indicated as is the sequence modification to introduce Kozac's consensus sequence (double underline). The bold portion was eliminated as an in RNA destabilizing sequence.

FIG. 3 shows the amino acid sequence of *E. coli* GDH enzyme expressed in both the tobacco and corn.

FIG. 4 shows a linear map of the plasmid vector pBI121:GDH1 developed in Example I. The plasmid has the uidA gene removed and the *gdhA* gene inserted.

FIG. 5 shows a circular map of the plasmid vector pUBGP1 used in the examples as starting material and a control for plasmids useful in *Zea mays*.

FIG. 6A shows the DNA sequence of the mutagenized *gdhA* gene for plant expression (tobacco and corn).

FIG. 6B shows the DNA sequence including the SphI of the mutagenized *gdhA* gene for plant expression (tobacco and corn).

FIG. 7A shows the mutagenized *gdhA* gene with the added restriction sites for use in *Zea mays*.

FIG. 7B shows a linear plasmid map of pBI 121::SSU::GDH1.

FIG. 8 shows the 3' EcoRI SphI adapter between nosT and plasmid for corn transformation.

FIG. 9 shows a circular map of the plasmid pUBGDH1 wherein UB is ubiquitin.

FIG. 10 shows a circular map of the plasmid vector pUBGDHI with the pre SS unit.

FIG. 11 shows the methylammonium uptake of tobacco transformants.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to methods of producing transgenic plants containing the *gdhA* gene. The term transgenic plant refers to plants having exogenous genetic sequences which are introduced into the genome of a plant by a transformation method and the progeny thereof.

Transformation Methods - are means for integrating new genetic coding sequences by the incorporation of these sequences into a plant of new genetic sequences through man assistance.

Though there are a large number of known methods to transform plants, certain types of plants are more amenable to transformation than are others. Tobacco is a readily transformable plant. The basic steps of transforming plants are known in the art. These steps are concisely outlined in U.S. patent number 5,484,956 "Fertile Transgenic *Zea mays* Plants Comprising Heterologous DNA Encoding Bacillus Thuringiensis Endotoxin" issued January 16, 1996 and U.S. patent number 5,489,520 "Process of Producing Fertile *Zea*

mays Plants and Progeny Comprising a Gene Encoding Phosphinothricin Acetyl Transferase" issued February 6, 1996.

1. Plant Lines

Plant cells such as maize can be transformed by a number of different techniques. Some of these techniques which have been reported on and are known in the art include maize pollen transformation (See University of Toledo 1993 U.S. Patent No. 5,177,010); Biolistic gun technology (Se U.S. patent number 5,484,956); Whiskers technology (See U.S. patent numbers 5,464,765 and 5,302,523); Electroporation; Agrobacterium (See 1996 article on transformation of maize cells in Nature Biotechnology, Volume 14, June 1996) along with numerous other methods which may have slightly lower efficiency rates then those listed. Some of these methods require specific types of cells and other methods can be practiced on any number of cell types.

The use of pollen, cotyledons, meristems and ovum as the target issue can eliminate the need for extensive tissue culture work. However, the present state of the technology does not provide very efficient use of this material.

Generally, cells derived from meristematic tissue are useful. Zygotic embryos can also be used. Additionally, the method of transformation of meristematic cells of cereal is also taught in the PCT application WO96/04392. Any of the various cell lines, tissues, plants and plant parts can and have been transformed by those having knowledge in the art. Methods of preparing callus from various plants are well known in the art and specific methods are detailed in patents and references used by those skilled in the art.

Cultures can be initiated from most of the above identified tissue. The material used herein was zygotic

embryos. The embryos are harvested and then either transformed or placed in media. Osmotic cell treatments may be given to enhance particle penetration, cell survival, etc.

The only true requirement of the transformed material is that it can form a fertile transformed plant. This gene can be used to transform a number of plants both monocots and dicots. The plants that are produced as field crops are particularly useful. These crops include cotton, corn, soybeans, sorghum, brassica, sunflower and some vegetables. The *gdhA* gene can come from various non-plant genes (such as; bacteria, yeast, animals, viruses). The *gdhA* gene can also come from plant gene. The gene insert used herein was either an *E. coli* glutamate dehydrogenase gene or a mutagenized version thereof.

The DNA used for transformation of these plants clearly may be circular, linear, double or single stranded. Usually, the DNA is in the form of a plasmid. The plasmid usually contains regulatory and/or targeting sequences which assists the expression of the gene in the plant. The methods of forming plasmids for transformation are known in the art. Plasmid components can include such items as: leader sequences, transit polypeptides, promoters, terminators, genes, introns, marker genes, etc. The structures of the gene orientations can be sense, antisense, partial antisense, or partial sense: multiple gene copies can be used.

The *gdhA* gene can be useful to change or alter the nitrogen assimilation pathway or to assist in the identification and/or heartiness of transformed material in the presence of herbicide. Clearly, the bar gene from *Streptomycin hygroscopicus* which encodes phosphinothricin acetyl transferase is resistance to phosphinothricin, and

bialaphos herbicid s (se U.S. patent 5,484,956, Table 1). Thus, this gene is useful as a selectabl marker gene.

Surprisingly, the present gene is tolerant to some levels of phosphinothricin and bialaphos though in th constructs tested, the present gene may evidence slightly more susceptibility to herbicide damage at high herbicide concentration then plants transformed with the bar and PAT genes. However, when the *gdhA* gene is combined with the PAT and/or bar gene, the transformed cells and/or plants have increased regenerability and heartiness after herbicide selection.

The regulatory promoters employed in the present invention can be constitutive such as CaMv35S for dicots and polyubiquitin for monocots or tissue specific promoters such as CAB promoters, etc. The prior art promoter include but is not limited to octopine synthase, nopaline synthase, CaMv19S, mannopine synthase. These regulatory sequences can be combined with introns, terminators, enhancers, leader sequences and the like in the material used for transformation.

The isolated DNA is then transformed into the plant. Many dicots can easily be transformed with Agrobacterium. Some monocots are more difficult to transform. As previously noted, there are a number of useful transformation processes. The improvements in transformatio technology are beginning to eliminate the need to regenerate plants from cells. Since 1986, the transformation of pollen has been published and recently the transforma on of plant meristems have been published. The transformation of ovum, pollen, and seedlings meristem greatly reduce the difficulties associated with cell regeneration of different plants or genotypes within a plant can present.

The most common method of transformation is referred to as gunning or microprojectile bombardment. This biolistic process has small gold coated particles coated with DNA shot into the transformable material. Techniques for gunning DNA into cells, tissue, callus, embryos, and the like are well known in the prior art.

After the transformation of the plant material is complete, the next step is identifying the cells or material which has been transformed. In some cases, a screenable marker is employed such as the beta-glucuronidase gene of the *uidA* locus of *E. coli*. Thus, the cells expressing the colored protein are selected for either regeneration or further use. In many cases, the transformed material is identified by a selectable marker. The putatively transformed material is exposed to a toxic agent at varying concentrations. The cells which are not transformed with the selectable marker that provides resistance to this toxic agent die. Cells or tissues containing the resistant selectable marker generally proliferate. It has been noted that although selectable markers protect the cells from some of the toxic affects of the herbicide or antibiotic, the cells may still be slightly effected by the toxic agent by having slower growth rates. The present invention is useful as a selectable marker for identifying transformed materials in the presence of the herbicide phosphinothricin. In fact, when combined with the PAT or bar gene which is known to give resistance to phosphinothricin, the cells or plants after exposure to the herbicide often evidences increased growth by weight and appear more vigorous and healthy.

If the transformed material was cell lines then these lines are regenerated into plants. The cell's lin· are treated to induce tissue differentiation. Methods of regeneration of cellular material are well known in the art

since early 1982. The plants from either the transformation process or the regeneration process are transgenic plants.

The following non-limiting examples are shown to mor particularly describe the present invention.

The DNA sequence of the *gdhA* gene of Escherichia coli which encodes a 447 amino acid polypeptide subunit of NADP-specific glutamate dehydrogenase was presented in 1982 in Nucleic Acids Research, Volume II, Number 15, 1983. Th present examples will illustrate the *gdhA* gene transformed into both dicot and monocot plants.

Example I

Fertile transgenic tobacco plants containing an isolated *gdhA* gene was prepared as follows:

A. The tobacco tissue for transformation was initiated and maintained.

Seed from *Nicotiana tabacum* var. Petite Havana were surface sterilized and germinated on MSO medium (Murashige and Skoog 1962). Two weeks after germination, leaves were excised and used in transformation experiments.

B. Formation of the Plasmid.

A bacterial glutamate dehydrogenase (*gdhA*) gene, shown in Fig. 1, derived from *E. coli*, was altered for expression in plant cells by polymerase chain reaction. The 5' non-coding region was modified by the introduction of an XbaI restriction enzyme site. Kozac's consensus sequence (Lutcke et. al. 1987) was also added to the 5' region to allow high levels of expression in plant cells. The 3' non-coding region

was altered to stabilize the mRNA and ensure appropriate polyadenylation and a SacI restriction site was added. These primer sequences, shown in Fig. 2, are the introduction of the restriction sites and the Kozac's consensus sequence along with the destabilizing portions. The amino acid sequence of the *gdhA* gene was retained. PCR was carried out in an automated thermal cycler (MJ Research, St. Louis, MO) for 25 cycles (each cycle consisting of 1 min. at 92° C, 1 min. at 60° C and 3 min. at 72° C). Reactions contained 200 ng of pBG1 (Mattaj et. al. 1981), 0.9 mM) $MgCL_2$, dNTPs, 1 unit of *Taq* polymerase (Promega, Madison, WI) and 1 nM of each primer. The PCR products were gel purified and DNA bands recovered from agarose gels using GeneClean (Bio101, Hercules, CA). XbaI and SacI were used with the band which was digested. This process provided single strand complementary end for ligation into a vector.

The *uidA* gene from pBI121.1 (pBI121 plasmid is commercially available from Clontech Laboratories, Palo Alto, CA), (Jefferson, 1987) was removed by restriction digest with XbaI and SacI and the gel eluted PCR products were ligated into the resulting 9.7kb fragment of pBI121.1. The amino acid sequence of the GDH enzyme produced by the *gdhA* gene is shown in Fig. 3. The plasmids were then transformed into competent *E. coli* cells (Top10 Invitrogen, San Diego, CA) via electroporation. Colony hybridization was used to detect colonies with the modified *gdhA* inserts (Fig. 3). Plasmids from the hybridizing colonies were used to transform competent Agrobacterium tumefaciens (Sambrook et. al. 1989) strains LBA4404 (Hooykas 1981) and EHA101 (Nester 1984).

C. Plant Transformation.

Nicotiana tabacum var. Petite Havana leaf discs from in vitro grown seedlings were transformed with the A. tumefaciens constructs using standard tobacco transformation procedures (Horsch et. al. 1988) with the following modification. Transformed shoots were selected on 300 µg/ml kanamycin. Shoots were excised and rooted in a sterile peat-based medium in GA7 vessels (Magenta Corp. Chicago, IL). The vessel lids were gradually removed (over 7-10 days) to acclimatize the plantlets to laboratory conditions before placement in the greenhouse.

D. Confirmation of Transformation with *gdhA* Gene.

To show that the tobacco has acquired the *gdhA* gene the specific activity of GDH was quantified by measuring the rate of oxidation of NADPH due to 2-oxoglutamate reductive amination. This enzyme assay was performed on cell free extracts.

1. Cell Free Extract Preparation

Leaf tissue (1-2g) was placed in 5 volumes of ice-cold buffer(200 mM Tris-HCL pH8.0, 14 mM).

2. Mercaptoethanol, 10 mM L-cysteine, 0.5 mM phenylmethylsulphonylfluroide, 0.5% (v/v) Triton x-100) [23]. Tissue was homogenized by Polytron (Tekmar, Cincinnati, OH) 4 times for 12 seconds each and was returned to an ice bath for 12 seconds between each grind. The slurry was centrifuged at 10,000g for 25 minutes and the supernatant was used for enzyme assays. *E. coli*

extracts were prepared as in Mountain et. al., 1985 [32]. This publication is hereby incorporated by reference. All steps were carried out at 4° C.

Gel Analysis and GDH Activity Staining

Regenerants were qualitatively tested for deaminating NADP-dependent GDH activity following gel electrophoresis of crude protein extracts after Lightfoot et. al., 1988. Electrophoresis of other protein extracts is known to those skilled in the art. Proteins were separated on a non-denaturing gel containing 5% polyacrylamide by electrophoresis for 2 hr at 120 V. NADP-specific GDH enzyme activity was visualized as a band in the gel by L-glutamate and NADP-dependent tetrazolium staining of GDH isozymes (50 mM Tris pH 9.3, 8 mg/ml glutamate, 0.04 mg/ml NADP, 0.04 mg/ml MTT, 0.04 mg/ml phenazine monosulphate and 0.08 mg/ml $CaCl_2$).

Enzyme assays

The specific activity of aminating NADPH-dependent GDH in cell free extracts was quantified by measuring the rate of oxidation of NADPH attributable to the reductive amination of 2-oxoglutarate. The reaction mixture initially consisted of 0.1 M Tris pH 8.5, 0.2M 2-α-ketoglutarate, ..0 MM $CaCl_2$, 0.2 mM NADPH, 200mM ammonium chloride and 50mM glutamine. The rate of change in absorption was measured at 340 nm for 1.5 mins before and 1.5 mins after the addition of

the 20mM or 200 mM ammonium chloride. Glutamine was then added to 5mM and the absorbance measured for a further 1.5 mins. Assays were performed at 25°C.

Glutamine synthetase activity was measured spectrophoto metrically by incubating the crude extract in a reaction mixture for 10 minutes by the transferase assay as taught in the art (see Cullinore J.V. Planta 150.39 2-396. 1980). The OD_{500} was measured, 1 µM γ-glutamyl hydroxamate has an OD_{500} of 0.4.

Glutamate concentration determination

Glutamate and glutamine concentrations were determined after separation on Dowex-1-acetate. Quantitation was by the ninhydrin spectrophotometric assay.

Table 1: Characteristics of Transgenic Plants

		Number of Lines	
Strain/Gene	Explants Inoculated	Antibiotic Resistant[a]	GDH[b]
EHA101/*gdhA*	30	17	12
LBA4404/*gdhA*	30	2	2

a = Resistant to 300 µg/ml Kanamycin® in an R_1 seedling assay.

b = Positive bands after electrophoresis of crude extract on 5% polyacrylamide gel followed by NADP-dependent tetrazolium staining of GDH isozymes.

Example II

The original plant transformation vector pBI121.1 was modified in Example I to contain the *gdhA* gene. In this example, the vector was unchanged and pBI121.1 containing *uidA* was used as the chimeric plasmid which was transformed into *E. coli* cells (Top10 Invitrogen, San Diego, CA) via electroporation. Colony hybridization was used to detect colonies with plasmids containing *uidA* gene. Plasmids from the hybridizing colonies were analyzed by single and double restriction digestions. Plasmids with the correct physical map were used to transform competent Agrobacterium tumefaciens strains LBA4404 and EHA101.

Plant Transformation.

Nicotiana tabacum var. Petite Havana leaf discs from in vitro grown seedlings were transformed with the A. tumefaciens constructs using standard tobacco transformation procedures as in the earlier example with the following modification. Transformed shoots were selected on 300 µg/ml kanamycin. Shoots were excised and rooted in a sterile peat-based medium in GA7 vessels (Magenta Corp. Chicago, IL). The vessel lids were gradually removed (over 7-10 days) to acclimatize the plantlets to laboratory conditions before placement in the greenhouse. The R_0 plants were allowed to flower and self fertilize to produce the R_1 seed. R_1 seed were collected from individual plants and stored at 4° C.

Table 2: Characteristics of *uidA*

Strain/Gene	Explants Inoculated	Number of Lines: Antibiotic Resistant[a]	Number of Lines: GDH[b]
LBA4404/*uidA*	15	2	0
EHA101/*uidA*	15	4	0

a = Resistant to 300 µg/ml Kanamycin® in an R_1 seedling assay.

b = Positive bands after electrophoresis of crude extract on 5% polyacrylamide gel followed by NADP-dependent tetrazolium staining of GDH isozymes.

Discussion of Examples I and II.

A non denaturing polyacrylamide gel containing bands produced from NADP-dependent staining of crude extracts of *E. coli*, *gdhA* transformed lines and one *uidA* line was performed and read. As expected, the *uidA* transformed line did not produce bands when stained with $NADP^+$ as the oxidant. Fourteen of the 19 antibiotic resistant *gdhA* transformants showed GDH activity as did the *E. coli*.

Table 3

Specific activity of NADPH-dependent GDH and ATP dependent GS in cell-free extracts of transgenic tobacco leaves.

Tobacco Line	Transforming Gene	GDH Activity NADPH Oxidation nM/mg*/min	GS activity nM/mg/min
2A	*gdhA*	2046	38
8	*gdhA*	1600	71
9	*gdhA*	1063	85
7B	*uidA*	0	85
E. coli	*gdhA*	215	59

* = Specific activity per mg of soluble protein.

Enzyme Specific Activity of Examples I and II.

High specific activities of GDH in *gdhA* transformed R_0 tobacco leaves were observed. The *gdhA* transformed tobacco lines produced up to 10 times more activity than *gdhA* in *E. coli*. NADP-specific GDH activity was not detectable in the *uidA* transformed tobacco lines.

GS activity was somewhat reduced in leaves of plant lines where the GDH activity was more than about 1100 nM/mg protein/min. The GDH activity was about 15-50 fold greater than the GS activity in the cell free extracts with saturating substrate concentrations. The GDH activity was not greatly reduced in assays containing 20 mM ammonium (data not shown) close to physiological NH_4 concentrations. Therefore, *gdhA* transformed plants may be assimilating ammonium at a rate equivalent to, or better than, GS.

The specific activity of GDH in cell free extracts show *gdhA* gene in plants at 5-10 times the *E. coli gdhA* activity. This was surprising as there was initially some question as to whether the bacterial gene would express well in the plant genome. The *gdhA* gene in plants have a GDH activity that is 15-50 times greater than the GS activity. Increased ammonium assimilation is apparently provided by GDH activity if substrate concentrations are not limiting.

Ammonium assimilation by GDH is energetically favorabl compared to GS since there is a net saving of one ATP. In addition, the higher specific activity of GDH might requir the synthesis of 10 fold fewer enzyme molecules per mole of ammonium assimilated.

Example III

Fertile transgenic tobacco containing *gdhA* gene and chloroplast transit peptides:

The plasmid constructed in Example I (shown in Fig. 4) does not target the *gdhA* gene to the area of tissue that it is presumed to be most helpful. The chloroplasts of the plant tissue is targeting in the present example. The pBI121 *gdhA* plasmid was modified to allow fusion with cleavable preprotein sequences (often referred to as chloroplast transit peptide sequences) from RUBISCO SSU (rbcS) by introduction of the SphI site.

PCR amplification of *gdhA* from pBI121::GDH1 using the mutagenic primer.

Primer SPHGDH5

GGT TTT ATA TgC ATg CAT CAg ACA TAT TC

5' SphI adapter for igation of *gdhA* with chloroplast targeting pre-peptide encoding sequences.

And the addition of the specific primer HUGDH3 (shown in Fig. 6B) was completed. The amplified 1.3 kbp fragment was subject to restriction digestion with SphI and SacI. Digestion of pBI121 with SmaI and SacI allowed recovery of the vector minus GUS (*uidA*) as a 9.6 kbp fragment. PCR amplification from the plasmid pPSR6 (Cashmore et. al., 1983) and restriction digestion allowed recovery of the preprotein encoding sequence as a 0.2 kbp fragment SmaI to SphI fragment. The 9.6 kbp pBI121 fragment was ligated with the 1.3 kbp fragment from pBI121::GDH1 and the 0.2 kbp fragment from pPRS6 to give pBI121::SSU::GDH1 (shown in Fig. 7) which was amplified in *E. coli* DH5.

Results of Examples

The transformed tobacco plants, leaves and seed Examples I and II were analyzed for percentage of nitrogen, protein and crude fat with the following result:

Table 4:

Tobacco Leaf Analysis

	% N	% Protein
uidA transformed	6.98	43.6
gdhA transformed	8.01	50.0

Tobacco Seed Analysis

	% N	% Protein	Crude Fat
uidA transformed	4.2	26.5	36.8
gdhA transformed	3.56	22.0	35.07
nontransformed	3.98	25.0	38.5

The leaf analysis shows a 1% nitrogen increase and a 6% increase in protein in the *gdhA* transformed plant. The seed analysis appears to indicate that the *gdhA* gene may be altering the accumulation of nitrogen, protein and crude fat in the tobacco seed.

Example IV

Ammonium Toxicity

The transformed tobacco seeds of the previous examples were used in an ammonium toxicity study. Ammonium toxicity was measured by germinating transformed tobacco seed on agar solidified MS media while excluding all nitrogen sources except ammonium chloride. The medium was supplemented with 10, 30, 50, 70 or 100 mM ammonium chloride but no nitrates. The seedlings were grown either with or without 30 mg/l sucrose. Ten to fifteen R_1 seeds were initiated per plat with four replications per concentration. Fresh and dry weights of 10 seedlings per plate were measured after six weeks on these media. Table 5 shows these results.

Table 5

Effect of concentration of ammonium chloride and genotype on dry weight of *gdhA* or *uidA* transformed R_1 tobacco seedlings. N_2 carbon source supplied.

	Dry weight (mg) of Transformed Lines											
NH_4^+ Conc.	2A (*gdhA*)	n*	B_2 (*gdhA*)	n	9_1 (*gdhA*)	n	7B (*uidA*)	n	Significance**	LSD* values 5%	1%
10mM	0.75	40	1.1	29	0.48	39	0.85	20	**	0.25	0.33
30	1.2	34	0.8	36	0.57	36	0.6	39	**	0.31	0.40
50	0.6	30	0.36	39	0.38	28	0.4	38	**	0.14	0.19
70	0.36	35	0.35	40	0.21	40	0.25	38	**	0.08	0.11
100	0.19	36	0.18	29	0.14	40	0.17	40	**	0.06	0.07
Significance	**		**		**		**				
LSD value											
5%	0.28		0.21		0.10		0.10				
1%	0.36		0.28		0.13		0.14				

*n = number of seedlings

** = significant results at the 1% level, NS = nonsignificant

LSD = Least Significant Difference as calculated by $T_\alpha(MSE/n)^{1/2}$

Increased resistance to ammonium chloride is partial as the GDH activity would affect primarily the nitrogen assimilation rate. Increased resistance to ammonium chloride is evident by the increase in fresh and dry weight accumulated by the *gdhA* transformed lines.

Example V

Field Traits of Transgenic Tobacco

The transformed tobacco was planted in a field and fertilized with 150 lb. per acre of ammonium nitrate. The following data on the field traits was collected.

Table 6

Mean field traits of transgenic tobacco fertilized with 150 lb. per acre ammonium nitrate.

Plant Line	NADPH-GDH Activity (nM/mg/min)	Dry Weight (g)	Nitrogen Content (%)	Height (cm)	Leaf Number	Leaf Length (cm)
91	1063	430	4.18	41.9	16.9	25.4
2A	2046	356	4.14	37.7	14.2	23.3
7B	0	288	4.14	41.2	13.6	23.3
BAR	0	154	4.16	36.4	12.9	22.8
LSD (0.05)		193	0.08	2.2	1.2	0.7

7B = *uidA* gene
2A = *gdhA* gene
91 = *gdhA* gene
BAR = Bar gene

If the control is the *uidA* gene in the transformed tobacco plants then the significant differences are in the leaf number and the leaf length between the 91 line and th 7B line. The Bar data across the chart, with the sole exception of the nitrogen content, is lower then the 7B line. It is within the LSD. If Bar is used as the control, dry weight and plant height (yield) is also significantly greater for Line 91.

Example VI

Construction of Plasmids to transfer *E. coli gdhA* to *Zea mays*.

The pBI121::GDH plasmid (shown in Fig. 4) was not particularly suitable for use in *Zea mays*. Thus, the plasmid pUBGPI (shown in Fig. 5) which is a vector suitable for transformation of *Zea mays* and foreign gene expression was employed.

The modified *E. coli gdhA* gene (shown in Fig. 6) was readily transferred to pUBGP1 to replace the GUS (*uidA*) gene by restriction digestion, gel purification of appropriate fragments and ligation as follows. Digestion of pBI121::GDH (shown in Fig. 4) with XbaI and EcoRI allowed recovery of *gdhA*::nosT as a 1.6 kbp fragment. Ligation with EcoRI XbaI digested pUC18 produced the plasmid pUCGDH1 which was amplified in *E. coli* DH5. Digestion of pUCGDH1 with PstI and EcoRII allowed recovery of the *gdhA*::nosT as a 1.6 kbp fragment. This mutagenized *gdhA* gene with the added linker restriction sites is shown in Fig. 7. Digestion of pUBGP1 with NcoI and SphI allowed recovery of the vector minus GUS::nosT as the 1.0 and 5.6 kbp fragments. Digestion of the 1.0 kbp fragment with PstI removed one NcoI site (and an inappropriate ATG codon). The 1.0 and 5.6 kbp pUBGPi

fragments were ligated with the 1.6 kbp fragment from pBI121::GDH1 and an EcoRI/SphI adapter (shown in Fig. 8).

The 3' EcoRI SphI adapter is between nosT and the plasmid for corn transformation. This gives pUBGDH1 (shown in Fig. 9) which was amplified in *E. coli* DH5.

The plasmid pUBGDH1 (shown in Fig. 9) was purified as DNA from *E. coli*, and 1µg were used for transformation of *Zea mays* inbred line H99 by biolistics.

Example VII

Construction of Plasmid to target the *E. coli gdhA* to chloroplasts in corn.

Because the pBI121::GDH plasmid was not suitable for *Zea mays* transformation or gene expression, another plasmid vector was used to achieve *gdhA* gene transfer and expression. The 1.8 kbp *Sma*I to *Eco*RI fragment of pBI121::SSU::GDH1 was isolated and ligated with an EcoRI/SmaI adapter and SmaI digested pUC18. This produced the plasmid pUCSSUGDH1 which was amplified in *E. coli* DH5. Digestion of pUCSSUGDH1 with SmaI allowed recovery of the SSU::*gdhA*::nosT as a 1.8 kbp fragment (Fig. 10). Digestion of pUBGP1 with *Nco*I and *Sph*I allowed recovery of the vector minus GUS::nosT as the 1.0 and 5.6 kbp fragments. Digestion of the 1.0 kbp fragment with PstI removed the *Nco*I site (and an inappropriate ATG codon). The 1.0 and 5.6 kbp pUBGPI fragments were ligated with the 1.8 kbp fragment from pUCSSUGDH1 and an *Pst*I/*Sma*I adapter to give pUBSSUGDH1 (Fig.11) which was amplified in *E. coli* DH5.

The plasmid pUBSSUGDH1 was purified as DNA from *E. coli*, and 1µg can be used for transformation of the *Zea mays* inbred lineby any method.

Example VIII: Method of Biochemical Analyses of Herbicde Resistance

Biochemical Analyses of Transformed Plants of the Above Examples.

Herbicide Resistance

Phosphinothricin (PPT) resistance was tested by initiating *gdhA* transformed leaf discs from greenhouse grown R_o plants on MSO medium containing 1 mg/l BA, 0.1 mg/l NAA, 3% w/v sucrose and 7 g/l agar was supplemented with the herbicide Ignite™ at 0, 0.1, 1.0 or 10.0 mg/l active ingredient (a.i.) (5 replications of 1 cm^2 discs in individual culture tubes per concentration). Four weeks after initiation, cultures were photographed and the volume of leaf discs was measured.

R_1 Seed from *gdhA* transformed R_o plants were also tested for herbicide resistance by germination and growth on MSO medium containing 3% w/v Sucrose and 7 g/l Agar supplemented with 0, 3, 9, 27 or 81 mg/l a.i. Ignite™ (30 seeds per plate with 3 replications per concentration) or 0, 1, 3, 10, 30 mg/l as noted in the text. Cultures were maintained at 25°C with 16 hours of light. Four weeks after germination, cultures were photographed.

Th *gdhA* transformed R_0 plants were also tested for herbicide resistance by painting leaves with 0, 3, 9, 27 or 81 mg/ml a.i. Ignite™ . Plants were maintained in the greenhouse. Four days after application chlorosis was scored.

Ammonium Toxicity Resistance

Resistance to ammonium toxicity in the absence of nitrate was measured by germinating transformed tobacco seed on agar solidified MSO medium excluding nitrogen. The medium was supplemented with 10, 30, 50, 70 or 100 mM ammonium chloride and seedlings were grown either with or without 30 mg/l sucrose. Ten to 15 seed were initiated per plate with 4 replications per concentration. Fresh and dry weights of 10 seedlings per plate were measured after 6 weeks on these media. Statistical analyses of these data were performed using SAS (SAS Institute Inc. Cary, NC).

The *gdhA* transformed R_0 plants were also tested for ammonium resistance by painting leaves with 100, 300, 500, 700 or 1000 mM ammonium chloride. Plants were maintained in the greenhouse. Four days after application chlorosis was scored.

Table 7

Mean growth traits of transgenic corn in the greenhouse.

Plant Line	NADPH-GDH Activity (nM/mg/min)	Fresh Weight (g)	Nitrogen Content (%)	Height (cm)
LL8	100	360	4.18	43.7
LL2	200	430	4.16	46.8
DL1	0	190	4.14	36.2
DL2	0	160	4.14	31.4
LSD (0.05)		160	0.10	5.2

LL8 = *gdhA* transformant of corn
LL2 = *gdhA* transformant of corn
DL1 = *uidA* transformant of corn
DL2 = *uidA* transformant of corn

The height of the *gdhA* transformant corn is significantly different then the DL1 and DL2 lines as are the fresh weights in grams. However, the nitrogen content is similar. The *gdhA* gene appears to be efficient in increasing the plant growth.

Table 8

Glutamate and Glutamine concentration in tobacco and corn roots expressing *gdhA*.

Plant Line	NADPH-GDH Activity (nM/mg/min)	Glutamate Concentration (uM/gfw)	Glutamine Concentration (mM)
a, Tobacco			
BAR	0	1.0	0.5
2A	2046	1.4	0.6
b. Corn			
DL1	0	1.1	0.8
LL1	800	1.3	0.9
LSD (0.05)		0.1	0.1

BAR = bar gene transformant of tobacco
2A = *gdhA* gene transformant of tobacco
DL1 = *uidA* gene transformant of corn
LL1 = *gdhA* gene transformant of corn

In each case, the *gdhA* transformants have increased th glutamate concentration in the plant roots significantly. The glutamine concentration also appears raised though not significantly.

Table 9

Effect of concentration of ammonium and genotype on fresh weight of *gdhA* and *uidA* transformed corn callus. No carbon source added.

NH4+ Conc.	LL2 (*gdhA*)	LL8 (*gdhA*)	DL1 (*uidA*)
10mM	22.2	25.6	23.1
30	29.3	27.8	16.0
50	15.8	12.6	8.5
70	9.9	9.3	7.7
100	8.3	6.4	6.2

Clearly, the *gdhA* transformed lines have a greater fresh weight then does the *uidA* lines. This indicates the *gdhA* activity is increasing cell proliferation.

Example IX

I. Uptake Experiments

Seeds from *gdhA* transformed plants and seeds from *uidA* transformed plants were germinated on MSO medium without nitrogen. The medium was supplemented with 4% w/v sucrose. Two weeks after germination, the nitrogen starved seedlings were used to test whether the *gdhA* transformed seedlings were capable of absorbing radiolabelled methylammonium at a greater rate than the *uidA* transformed control plants. Fifteen seedlings were floated in the treatment solution (0.2mM $CaCl_2$, 0.2 mM Mes pH 6.0, and 200 µM KCl) for 10 minutes. Radiolabelled 14C-methylammonium was then added to

the treatment solution at a concentration of 1 mM. After 12, 24, 36, 48 or 60 minutes the labeled solution was aspirated and replaced with nonlabeled solution. The wash solution was aspirated after 2 minutes and the seedlings were transferred to scintillation vials. The seedlings were ground in 1 ml of water for 2 min. with a polytron (Tekmar Cinn. OH) to break open the cells. 2 mls of scintillation fluid was added per vial. The radioactivity absorbed by each sample was counted using an LS6000 scintillation counter (Beckman, CA) with an open window.

As indicated above in the previous example, the biochemical analysis of methylammonium uptake was tested. The transformed tobacco developed under the first couple of examples were employed in the uptake study. The results are shown in Fig. 11. The uptake of both the *uidA* and *gdhA* lines without 1mM NH_4 was greatly enhanced in the time frame given.

II. Herbicide Resistance

A surprising aspect of the present gene in plant transformants is its tolerance to the herbicide phosphinothricin. The addition of the *gdhA* gene to either the PAT gene or the Bar gene apparently provides the plant with added resistance as shown by the plants' ability to continue to flourish and grow in increasing concentrations of herbicide. There are a number of commercially available herbicides that fit within the class of phosphinothricin herbicides.

The tobacco transformants of *gdhA* and *uidA* do not carry either the bar nor the PAT gene. A control used for comparison was a tobacco transformant containing the Bar gene. In contrast, the corn transformants all contain the

PAT gene as th selectable marker. Therefore, the corn transformants show that the combination of phosphinothricin resistant gene(s) such as PAT in combination with the *gdhA* gene provides plants with increased resistance to chlorosis.

Example X

GDH activity of *gdhA* transformants and controls.

The tobacco transformants including the Bar transformant were developed either in examples provided earlier or by similar methods. The biochemical analysis was performed as indicated above. The results are as follows:

Table 10

Characteristics of the tobacco transgenic plant lines recovered.

Strain/Gene	Explants Inoculated	*gdhA*[a]	PPT[b]
EHA101/*gdhA*	30	12	10
LBA4404/*gdhA*	30	2	1
LBA4404/*gdhA*	15	0	0
EHA101/*uidA*	15	0	0
LBA4404/bar	15	0	4

[a] = positive bands after electrophoresis of crude extract on 5% polyacrylamide gel followed by NADP-dependent tetrazolium staining of GDH isozymes.

[b] = Seedlings resistant to 3 µg a.i./ml PPT.

Clearly, both the LBA4404/bar and EHA100/*gdhA* lines as seedlings were resistant to 3 µg a.i./ml PPT. Thus, the tobacco plants can be sprayed in a field with weeds with the PPT herbicide and at least at the indicated levels of PPT will not have chlorosis evidenced.

Example XI

Volume of tobacco callus formed in present of various levels of PPT. The transformants of the earlier examples were tested in various herbicide concentrations.

Table 11

Mean volume of tobacco callus* with various concentrations of the herbicide Ignite™ (PPT).

Herbicide conc. (g ai/ha)	Transformed Lines								
	2A (*gdhA*) (cm^3)	n°	8; (*gdhA*) (cm^3)	n	9, (*gdhA*) (cm^3)	n	7B (*uidA*) (cm^3)	n	Significance'
0.0	20929.4**	5	17275.0	5	20761.2	5	16056.9	3	NS
0.1	12873.2	5	11489.0	3	14949.5	4	2515.9	2	**
1.0	3828.7	4	6478.1	5	9634.0	5	0.0	4	**
10.0	0.0	5	0.0	5	0.0	5	0.0	4	NS

*Callus tissues grown leaf explant from *gdhA* or *uidA* R_0 transformed plants were initiated on MS medium in Petri dishes and incubated in the light at 25°C for 4 weeks before volume was calculated.

n = number of explants

** = significant at the 1% level, NS = nonsignificant

'Volume was calculated using the formula 3.14r²h

The evidence clearly indicates that the volume of callus of *uidA* tobacco callus in PPT is significantly less then callus of the *gdhA* tobacco.

Example XII

Volume of Corn Callus in Present of PPT

The volume of corn callus by volume was calculated in light of different transformant lines. Unlike the previous example, there is no control line that does not carry a PAT gene. Both the *gdhA* and the *uidA* transformants contain PAT which has resistance to PPT.

2180786

Table 12

Mean volume of corn callus[a] with various concentrations of the herbicide Ignite™ (PPT).

	Transformed Lines								
Herbicide conc. mg a.i./l	LL1 (gdhA) (cm^3)	n[b]	LL2 (gdhA) (cm^3)	n	LL4 (gdhA) (cm^3)	n	DL1 (uidA) (cm^3)	n	Significance[c]
10.0	22000 a	5	18000	5	19000	5	20000	3	NS
20.0	14000	[illegible]	12000	[illegible]	15000	4	2500	2	**
30.0	1000	4	6500	5	5000	5	0	4	**
40.0	0	5	0	5	0	5	0	4	NS

[a] Greenhouse grown leaf tissue from gdhA or uidA R_0 transformed plants were initiated on MS medium in culture tubes and incubated in the light at 25°C for 4 weeks before volume was calculated.

[b] n = number of explants

[c] ** = significant at the 1% level, NS = nonsignificant

[d] Volume was calculated using the formula $3.14r^2h$

Clearly, the *gdhA* transformants are contributing additional resistance to PPT herbicides beyond that conferred by the PAT gene alone.

Example XIII

R0 Plants Herbicide Resistance

The results of herbicide resistance in R_0 corn and tobacco transformants and the *gdhA* activity as measured by NADPH-GDH was compared. The following results were gathered.

Table 13

Herbicide resistance concentration dependence and *gdhA* activity in RO plants expressing *gdhA*.

Plant Line	NADPH-GDH Activity (nM/mg/min)	PPT concentration (mg a.i /ml)				
		0	1	3	10	30
a, Tobacco						
uidA	0	+	-	-	-	-
2A (*gdhA*)	2046	+	+	+	+	-
32(*gdhA*)	1600	+	+	+	+	-
91(*gdhA*)	1000	+	+	+	+	-
64(*gdhA*)	800	+	+	+	-	-
52(*gdhA*)	200	+	+	-	-	-
b, Corn	(all contain PAT gene)					
LL8(*gdhA*)	100	+	+	+	+	-
LL2(*gdhA*)	200	+	+	+	+	+
DL1(*gdhA*)	0	+	+	+	-	-
DL2(*gdhA*)	0	+	+	+	-	-

The results show that transformants without the *gdhA* gene provide no protection against the herbicide. The transformant 52 evidences the least amount of NADPH-GDH activity and it still gives resistance at 1 mg. a.i./ml of PPT.

Activity levels of NADPH-GDH of 1000 and over provide PPT resistance in tobacco. In corn, which has the added PPT resistance, the controls were not resistant after 3 mg. a.i./ml. However, activity levels of 100 of NADPH-GDH raised the tolerance to 10 mg. a.i./ml. The combination of the *gdhA* gene which expresses well and the PAT gene in corn shows even 30 mg a.i./ml of PPT can be resistant (LL2).

Example XIV

Progeny of corn plants containing *gdhA* gene and either the *Bar* gene or the *PAT* gene which are bred and developed from the seeds of the R_0 plants of the examples above can be planted in a field. This field could then be sprayed for weeds with a phosphinothricin herbicide such as Ignite™. This is a method of increasing plant growth. This herbicide spraying would eliminate most of the undesirable vegetation and the plants containing the *gdhA* gene would survive and increase growth. Alternatively, the corn plants can be transformed with the *gdhA* gene only and not include the selectable marker of either *Bar* or *PAT*. This transformant would be expected to survive the spraying and also show increased growth though it may be slightly less tolerant.

The *gdhA* gene can be transformed into crop plants that would not be expected to be effected by the herbicide PPT.

This would allow better growth of these plants in fields that are sprayed or in those that are not sprayed.

Example XV

Improved crop nitrogen assimilation can reduc environmental contamination by nitrates. Specialty corn hybrids for planting in watershed areas or for biofuel feedstocks will be developed.

Nitrogen Runoff Determinations

Plants were fertilized with 1 liter of 10mM ammonium nitrate and subsequently not watered or fertilized. After 48 hours, the root system was flushed with 10 liters of water and the runoff water from each pot collected. The ammonia concentration in each run-off water sample was determined by Nesslerization. Briefly, 1 ml. of sample was mixed with 1 ml. of 0.2% gum acacia solution, 1 ml. of Nesslers reagent, 7 ml. of water. After 20 minutes, the absorbance was determined at 420 nm. The nitrite concentration was determined by mixing 2 ml. of sample. 5 ml. of sulphanilic acid solution and 5 ml. of alpha-napathyl amine solution. After 30-60 minutes, the absorbance was determined at 540 nm. The nitrate concentration was determined by the 4-methylumbelliferone method. Briefly, 0.5 ml. of sample was mixed with 50 µl of 1 M sulfamic acid and heated to 100 C for 5 minutes. On ice 10 ml of 4.4 M ammonia. After 20 minutes at room temperature, the absorbance was determined at 540 nm.

Table 14

Nitrogen runoff rate in tobacco and corn expressing *gdhA*.

Plant Line	NADPH-GDH Activity (nM/mg/min)	Ammonium Concentration (mM)	Nitrate Concentration (mM)	Nitrate Concentration (mM)
a. Tobacco				
BAR	0	0.3	0.3	0.4
2A	2046	0.2	0.1	0.2
b. Corn				
DL1	0	0.2	0.3	0.5
LL1	800	0.1	0.1	0.2

These results show that the *gdhA* gene can be used to decrease the nitrogen content of runoff-water. The increased assimilation by plant roots results in less nitrogen to be available for leaching.

Significance

Biofuels/Watershed Premium

Unassimilated nitrogen is converted to nitrate and much is leached from the soil and into groundwater. The EPA is already considering setting limits on nitrogen use in watershed areas. Agricultural inputs contribute to nitrogen contamination in Illinois drinking water, particularly in the North Central region. More than 1? community water supplies and 25% of the 360,000 private wells contain concentrations of nitrogen above the EPA limits. Improving corn nitrogen assimilation with foreign transgenes may reduce nitrogen loss

by increasing assimilation. Attempts to dev lop such corn might be used to delay restrictive legislation and increas support for corn derived biofuels. Approximately 20% of Illinois farmland is in watershed areas. An increase of 10% in the corn derived ethanol as oxygenate addition to gasoline would double the demand for corn and would lead to high r corn prices.

Health Benefits

The association between dietary nitrates and several cancers is weak but positive (Moller et. al. 1990). Groundwater consumption can be a significant source of dietary nitrates in Illinois (Lee and Neilson 1987). Reducing groundwater contamination by nitrates may have a small beneficial effect on the rate of cancers. Infants 9-6 years old are at particular risk from dietary nitrates because nitrate reacts strongly with their blood hemoglobin causing methemoglobinemia, a condition similar to carbon monoxide poisoning in adults (Marschner, 1995). Bottled water is periodically recommended for infants in 18 Illinois communities with high nitrogen in their water supplies. Dietary nitrates are associated with higher abortion rates (Prins, 1983).

Environmental Premium

A health food or environmental premium on the market price of improved corn might be developed by marketing strategies. This might also lead to increased utilization. If a 1 cent per bushel premium for "low nitrogen impact" corn developed and Illinois farmers grew 1.74 billion bushels then profits would increase $17.4 million in Illinois.

Reduced Producer Losses

Assuming a 10% nitrogen loss, 175 lb/acre use, 10 cents/lb cost, and 13 million acres planted then income losses are: 0.1 x 175 x 10 x 13,000,000 = $23 million or $1.75 per acre. Although annual producer losses may approach $23 million per year in Illinois this is likely to vary depending on weather, soil types and cultural practices. The technology proposed might reduce producer expenses some part of that $23 million per year in Illinois.

Example XVI

Altered Seed Composition

Using the R_1 plants produced by the previous examples, the plants can be further modified to include genes that alter seed composition. A number of these types of genes are known in the art. These genes make altered hybrids. Altered seed composition leads to several specialty corn hybrids and products. High protein corn could be produced by increasing nitrogen assimilation. High sucrose corn or increased starch accumulation could be produced by simultaneous manipulation of carbon and nitrogen metabolism. The *gdhA* gene used in association with genes that alter starch content or chemical form or sugar content or form to promote alterations in plant composition.

WE CLAIM:

1. A method of improving crop growth which comprises:

applying to a field containing a crop, which are phosphinothricin resistant due to having an expressable transgene encoding for phosphinothricin resistant glutamate dehygrodenase enzyme a sufficient amount of a phosphinothricin class herbicide to control undesirable vegetation without significantly affecting crop growth.

2. A method according to claim 1 wherein said gene is mutagenized.

3. A method according to claim 1 wherein said gene is a modified bacterial gene.

4. A method according to claim 1 wherein said gene contains the Kozac consensus sequence.

5. A method according to claim 1 wherein said phosphinothricin class herbicide is combined with a second herbicide.

6. A method according to claim 1 wherein said crop is selected from the group consisting of corn, cotton, brassica, soybeans, wheat or rice.

7. Transgenic plant cells and progeny thereof comprising:

1) an expression cassettes having a transcription initiation region functional in the plant cells;

2) a DNA sequence that encodes for the GDH enzyme in said plant cells; and

3) a transcription termination region functional in said plant cells,

wherein said expression cassette imparts a detectabl level of herbicide resistance to the phosphinothricin class of herbicides.

8. Cells according to claim 7 wherein said at least one of said transcription region and said termination region is not naturally associated with said sequence.

9. Cells according to claim 7 wherein the sequence is from a bacterial gene.

10. Cells according to claim 9 wherein said bacterial gene is *E. coli*.

11. Cells according to claim 9 wherein said sequence from said bacterial gene is modified to enhance expression in plant cells.

12. Cells according to claim 7 wherein the DNA sequence encodes the amino acid sequence shown in Figure 3.

13. Cells according to claim 7 further comprising a chloroplast transient peptide adapted to target the chloroplasts.

14. Cells according to claim 7 wherein said transcription initiation region is constitutive in action.

15. Cells according to claim 7 wherein said transcription initiation region is organ specific.

16. A cell culture of cells according to claim 7 further comprising a marker gene that is capable of growth in a culture medium which includes a herbicide which is in the phosphinothricin class.

17. A cell culture of cells according to claim 7 further comprising a marker gene that is capable of growth in a culture medium which includes a herbicide which is not a phosphinothricin class.

18. A cell culture of claim 16 wherein said herbicide is bialaphos.

19. A transgenic plant originally formed from nontransgenic plants,, and progeny thereof which contains:

1) an expression cassette having a transcription initiation region functional in the plant cell;

2) a genetically engineered DNA sequence that is capable of encoding for the GDH enzyme in said plant cells;

3) wherein said plant evidences detectable increases in GDH activity when compared to said nontransgenic plants like that from which said transgenic plant was formed.

20. A transgenic plant according to claim 19 wherein said plant is a dicot.

21. A transgenic plant according to claim 20 wherein said plant is a monocot.

22. A transgenic plant according to claim 21 wherein said plant is *Zea mays*.

23. A transgenic plant according to claim 20 wherein said plant is selected from a group consisting of:

brassica, cotton, soybeans, tobacco.

24. A transgenic plant according to claim 19 wherein said plant forms seeds and said plant further comprising a genetically engineered DNA sequence that alters at least one of the protein and oil content of the seed of said plant and evidences altered GDH activity when compared to a transgenic plant containing said oil altering DNA sequence.

25. A transformed corn plant containing a bacterial glutamate dehydrogenase gene.

26. A recombinant plasmid characteristic in that the recombinant plasmid contains a constitutive promoter, a chloroplast transit peptide and the bacterial *gdhA* gene and transcriptional termination region.

27. A biologically pure culture of a bacterium characterized in that the bacterium is transformed with the recombinant plasmid of claim 26.

28. A transformed corn plant according to claim 25 further containing a second gene that was introduced into the plant or its ancestors by genetic engineering that is resistant to PPT.

Fig 1

2180726

E.coli gdhA

TCGAAAACTGCAAAAGCACATGACATAAACAACATAAGCACAATCGTATTAATATATAAGGGTTTTATA

TCTATGCATCACACATATTCTCTGCAGTCATTCCTCAACCATGTCCAAAAG

CGCGACCCGAATCAAACCGAGTTCGCCGCAAGCCGTTCGTGAAGTAATGACCACACTCTGCCCTTTTCTT

GAACAAAATCCAAAATATCCCCAGATGTCATTACTGGAGCGTCTGCTTGAA

CCGGAGCGCGTGATCCAGTTTCGCGTGGTATGGGTTGATGATCGCAACGCAGATACAGGTCAACCGTGCAT

GGCGTGTGCAGTTCAGCTCTGCCATCGGCCCGTACAAAGGCGGTATGCGC

TTCCATCCGTCAGTTAACCTTTCCATTCTCAAATTCCTCGGCTTTGAACAAACCTTCAAAAATGCCCTGA

CTACTCTGCCGATGGCGGTGGTAAAGCCCGCAGCGATTTCGATCCGAAA

GGAAAAAGCGAAGCTGAAGTGATGCGTTTTTGCCAGGCGCTGATGACTGAACTGTATCGCCACCTGGGCG

CGGATACCGACGTTCCGGCAGGTGATATCGGGGTTGGTGGTCGTCAAGTC

GGCTTTATGGCGGGGATGATGAAAAAGCTCTCCAACAATACCGCCTGCGTCTTCACCGGTAAGGGCCTTT

CATTTGGCGGCAGTCTTATTCGCCCCGAAGCTACCGGCTACGGTCTGGTT

TATTTCACAGAAGCAATGCTAAAACGCCACGGTATGGGTTTTGAAGGATGCGCGTTTCCGTTTCTGGCT

CCGGCAACGTCGCCCAGTACGCTATCGAAAAAGCGATGGAATTTGGTGCT

CGTGTGATCACTGCGTCAGACTCCAGCGGCAGTGTAGTTGATGAAACCGGATTCACGAAAGAGAAACTGGC

ACGTCTTATCGAAATCAAAGCCAGCCGCGATGGTCGAGTGGCAGATTAC

GCCAAAGAATTTGGTCTGGTCTATCTCGAAGGCCAACAGCGTGGTCTCTACCGGTTGATATCGCCCTGCCT

TGCGCCACCCAGAATGAACTGGATGTTGACGCCGCGCATCAGCTTATC

GCTAATGGCGTTAAAGCCGTCGCCGAAGGGGCAAATATGCCGACCACCATCGAAGCGACTGAACTGTTCGAC

CAGCCAGGCGTACTATTTGCACCGGGTAAAGCGGCTAATGCTGGTGGC

GTCGCTACATGGGGCCTGGAAATGGCACAAAACGCTCCCCCCCTGGCTGGCAAAGCCGAGAAAGTTGACGCA

CGTTTGCATCACATCATGCTGGATATCCACCATGCCTGTGTTGACCAT

GGTGGTGAAGGTCAGCAAACCAACTACGTGCAGGGCGCGAACATTGCCGGTTTTGTGAAGGTTGCCGATGCG

ATGCTGGCGCACGGTGTGATTTAAGTTGTAAATGCCTGATGGCGCTAC

GCTTATCAGCCCTACAAATGCGCACAATTCATTGCAGTTACGCTCTAATGTAGGCGCGCCAAGCGCAGCGCC

CCCGGCAAAATTTCAGCGCTTTATGAGTATTTAACGGATGATGCTCCC

Fig 1 (co..t'd) 2180736

CACGGAACATTTCTTATNGGCCAACGGCATTTCTTACTGTAGTGCTCCCAAAACTGCTTGTCGTAACGATAA

CACGCTTCAAGTTCAGCATCCGTTAAC

Long

a, Forward primer at 5'

```
                XbaI   Kozak
5'-...G GGT TCT AGA ACA ATG GAT CAG ACA TAT TCT CTG GAG...3'
```

```
                          start
                          codon
5'-...G GGT TTT ATA TCT ATG GAT CAG ACA TAT TCT CTG GAG TCA TTC CTC AAC-gdhA
3'-...C CCA AAA TAT AGA TAC CTA GTC TGT ATA AGA GAC CAC AGT AAG GAG TTC-gene
                           M   D   Q   T   Y   S   L   E   S   F   L   N
```

b, Reverse primer at 3'

```
                                                stop          mRNA
                                                codon    destabilizer
gdhA--T GCG ATG CTG GCG CAG GGT GAG ATT TAA GTT GTA AAT G...-5'
gene--C CGC TAC GAC CGC GTC CCA CTC TAA ATT CAA CAT TTA C...-3'
.........A   M   L   A   Q   G   V   I
```

```
3'...C TAC GAC CGC GTC CCA CAC TAA ATT CTC GAG TTA C...5'
                                       SacI
```

2180786

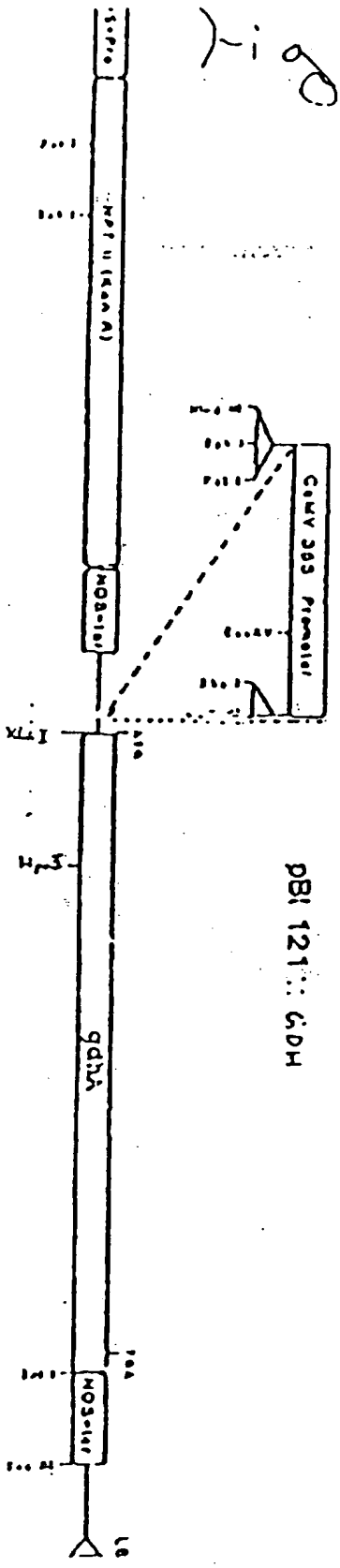
Fig 3. 2180786

Amino acid sequence of E.coli GDH enzyme expressed in plants (tobacco and corn).

METAspGlnThrTyrSerLeuGluSerPheLeuAsnHisValGlnLysArgAspProAsn
GlnThrGluPheAlaGlnAlaValArgGluValMETThrThrLeuTrpProPheLeuGlu
GlnAsnProLysTyrArgGlnMETSerLeuLeuGluArgLeuValGluProGluArgVal
IleGlnPheArgValValTrpValAspAspArgAsnGlnIleGlnValAsnArgAlaTrp
ArgValGlnPheSerSerAlaIleGlyProTyrLysGlyGlyMETArgPheHisProSer
ValAsnLeuSerIleLeuLysPheLeuGlyPheGluGlnThrPheLysAsnAlaLeuThr
ThrLeuProMETGlyGlyGlyLysGlyGlySerAspPheAspProLysGlyLysSerGlu
GlyGluValMETArgPheCysGlnAlaLeuMETThrGluLeuTyrArgHisLeuGlyAla
AspThrAspValProAlaGlyAspIleGlyValGlyArgGluValGlyPheMETAla
GlyMETMETLysLysLeuSerAsnAsnThrAlaCysValPheThrGlyLysGlyLeuSer
PheGlyGlySerLeuIleArgProGluAlaThrGlyTyrGlyLeuValTyrPheThrGlu
AlaMETLeuLysArgHisGlyMETGlyPheGluGlyMETArgValSerValSerGlySer
GlyAsnValAlaGlnTyrAlaIleGluLysAlaMETGluPheGlyAlaArgValIleThr
AlaSerAspSerSerGlyThrValValAspGluSerGlyPheThrLysGluLysLeuAla
ArgLeuIleGluIleLysAlaSerArgAspGlyArgValAlaAspTyrAlaLysGluPhe
GlyLeuValTyrLeuGluGlyGlnGlnProTrpSerLeuProValAspIleAlaLeuPro
CysAlaThrGlnAsnGluLeuAspValAspAlaAlaHisCysIleLeuAlaAsnGlyVal
LysAlaValAlaGluGlyAlaAsnMETProThrThrIleGluAlaThrGluLeuPheGln
GlnAlaGlyValLeuPheAlaProGlyLysAlaAlaAsnAlaGlyGlyValAlaThrSer
GlyLeuGluMETAlaGlnAsnAlaAlaArgLeuGlyTrpLysAlaGluLysValAspAla
ArgLeuHisHisIleMETLeuAspIleHisHisAlaCysValAspHisGlyGlyGluGly
GluGlnThrAsnTyrValGlnGlyAlaAsnIleAlaGlyPheValLysValAlaAspAla
METLeuAlaGlnGlyValIle

Fig 4

2180786



pBI 121:: GDH

Figure 5

2180783

UB-GUS-PAT Construct

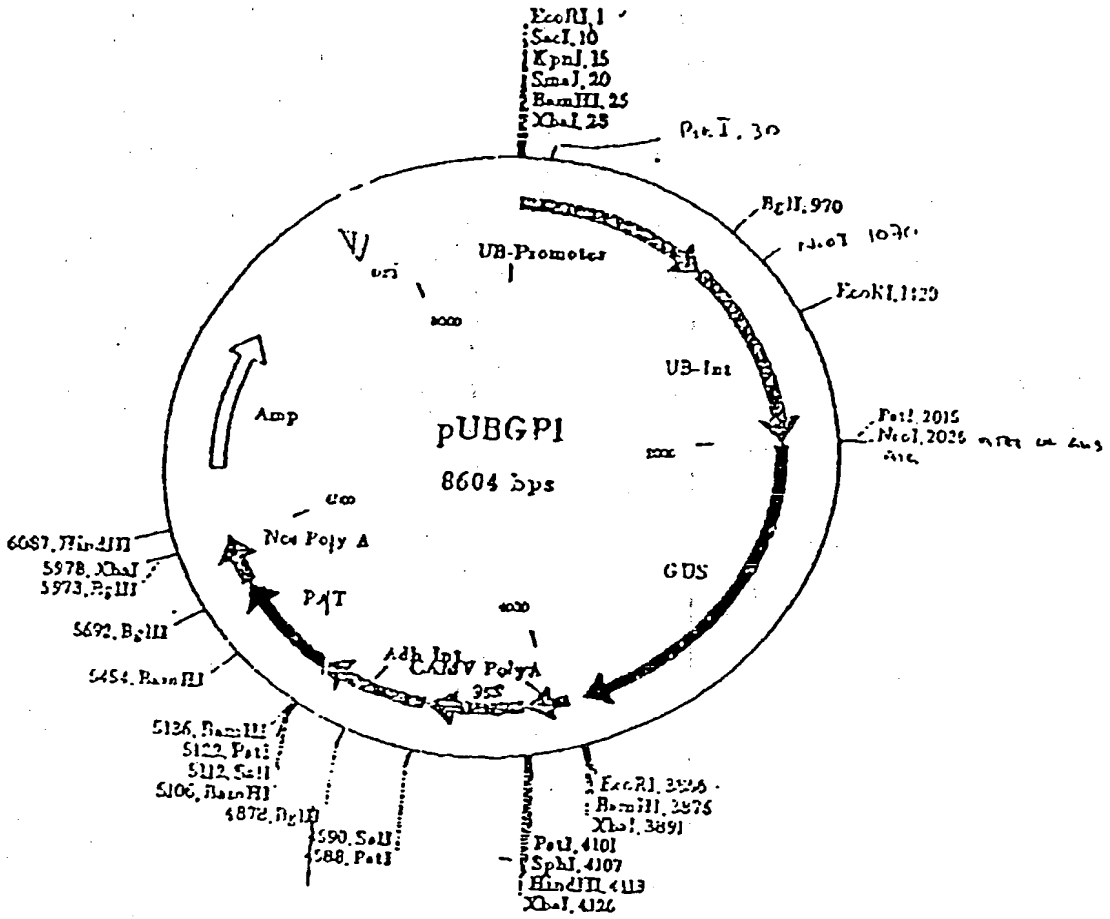


Fig 6A 2180786

Mutagenized gdhA for Plant expression (tobacco and corn)

XhoI Kozak

5'- TCTAGAACAATGCATCAGACATATTCTCTCGACTCATTCGTCAACCATCTCCAAAAG

CGCGACCCGAATCAAAGCCAGTTCGGGCAAGCCGTTCGTGAAGTAATCACCACACTCTGGCCCTTTTCTT

GAACAAAATCCAAAATATCGCCAGATGTCATTACTGGAGCGTCTGGTTGAA

CCGGAGCCGCGTGATCCAGTTTCGCGTGGTATGGGTTCATCATCGCAACCAGATACAGGTCAACCGTGCAT

GGCCTGTGCAGTTCAGCTCTGCCATCGGCCCGTACAAAGGCGGTATGCGC

TTCCATCCGTCAGTTAACCTTTCCATTCTCAAATTCCTCGGCTTTGAACAAACCTTCAAAAATGCCCTCA

CTACTCTGCCCATGGGCGGTGGTAAAGGCGGCAGCGATTTCGATCCGAAA

GGAAAAAGCGAAGGTGAAGTGATGCGTTTTTGCCAGGCGCTGATGACTGAACTGTATCGCCACCTGGCGG

CGGATACCGACGTTCCGGCAGGTGATATCGGGGTTGGTGGTCGTGAAGTC

GGCTTTATGGCGGGGATGATGAAAAAGCTCTCCAACAATACCCCCTGCGTCTTCACCGGTAAGCGCCCTTT

CATTTGGCCGCAGTCTTATTCGCCCGGAAGCTACCGGCTACGGTCTGGTT

TATTTCACAGAAGCAATGCTAAAACGCCACGGTATGGGTTTTGAAGGGATGCCGCGTTTCCGTTTCTGGCT

CCGGCAACGTCGCCCAGTACGCTATCGAAAAAGCGATGCAATTTGGTGCT

CGTGTGATCACTGCGTCAGACTCCAGCGGCACTGTAGTTGATGAAAGCGGATTCACGAAAGAGAAACTGGC

ACGTCTTATCGAAATCAAAGCCAGCCGCGATGGTCGAGTGGCAGATTAC

GCCAAAGAATTTGGTCTGGTCTATCTCGAAGGCCAACAGCCGTGGTCTCTACCGGTTGATATCGCCCTGCCT

TGCGCCACCCAGAATGAACTGGATGTTGACGCCGGCGATCAGCTTATC

GCTAATGGCGTTAAAGCCGTCGCCGAAGGGGCAAATATGCCGACCACCATCGAAGCGACTGAACTGTTCGAG

CAGGCAGGCGTACTATTTGCACCGGGTAAAGCGGCTAATGCTGGTGGC

GTCGCTACATCGGCCTGGAAATGCCACAAAACGCTGCGCGCCTGGGCTGGAAAGCCGAGAAAGTTGACGCA

CGTTTGCATCACATCATGCTGGATATCCACCATGCCTGTGTTGACCAT

GGTGGTGAAGGTGAGCAAACCAACTACGTGCAGGGCCCGAACATTGCCGGTTTTTGTGAAGGTTGCCGATGCG

ATGCTGGCGCAGGGTGTGATTTAAGTTGTAAATGCCTGATGGCGCTAC

GCTTATCAGGCCTACAAATGGGCACAATTCATTGCAGTTACGCTCTAATGTAGGCCGGGCAAGCGCAGCGCC

CCCGGCAAAATTTCAGGCGTTTATGAGTATTTAAGAGCTC

SacI

Fig 6B

2180786

Mutagenized gdhA for chloroplast targetting (tobacco and corn)

SphI

gCATgCATCAGACATATTCTCTGGAGTCATTGCTCAACCATATTCCAAAAC

1

CGCGACCCCAATCAAACCGAGTTCGCCCAAGCCGTTCGTGAAGTAATGACCACACTCTCGCCTTTTCTT

GAACAAAATCCAAAATATCGCCAGATGTCATTACTCGAGCGTCTGGTTGAA

CCGGAGCGCGTGATCCAGTTTCGCGTGGTATGGGTTCATGATCGCAACCAGATACAGGTCAACCCTGCAT

GGCGTGTGCAGTTCAGCTCTCCCATCGGCCCCGTACAAAGGCGGTATGCGC

TTCCATCCGTCAGTTAACCTTTCCATTCTCAAATTCCTCGGCTTTGAACAAACCTTCAAAAATGCCCTCA

CTACTCTGCCGATGGGCCGTGGTAAAGGCGGCAGCGATTTCGATCCGAAA

CGAAAAAGCGAAGGTGAAGTGATGCGTTTTTTCCCAGGCGCTGATGACTGAACTGTATCGCCACCTGGGCG

CCGATACCGACGTTCCGGCAGGTGATATCGGGGTTGGTGGTCGTGAAGTC

GGCTTTATGGCGGGGATGATGAAAAAGCTCTCCAACAATACCGCCTGCGTCTTCACCGGTAAGGGCCTTT

CATTTGGCGGCAGTCTTATTCGCCCGGAAGCTACCGGCTACGGTCTGGTT

TATTTCACAGAAGCAATGCTAAAACGCCACGGTATGGGTTTTGAAGGGATGCGCGTTTCCGTTTCTGGCT

CCGGCAACGTCGCCCAGTACGCTATCGAAAAAGCGATGGAATTTGGTGCT

CGTGTGATCACTGCGTCAGACTCCAGCGGCACTGTAGTTGATGAAAGCGGATTCACGAAAGAGAAACTGGC

ACGTCTTATCGAAATCAAAGCCAGCCGCGATGGTCGAGTGGCAGATTAC

GCCAAAGAATTTGGTCTGGTCTATCTCGAAGGCCAACAGCCGTGGTCTCTACCGGTTGATATCGCCCTGCCT

TGCGCCACCCAGAATGAACTGGATGTTGACGCCGCGCATCAGCTTATC

GCTAATGGCGTTAAAGCCGTCGCCGAAGGGGCAAATATGCCGACCACCATCGAAGCGACTGAACTGTTCGAG

CAGGCAGGCGTACTATTTGCACCCCCTAAAGCGGCTAATGCTGGTGGC

GTCGCTACATCGGGCCTGGAAATGGCACAAAACGCTGCGCGCCTGGGCTGGAAAGCCGAGAAAGTTGAGGCA

CGTTTGCATCACATCATGCTGGATATCCACCATGCCTGTGTTGACCAT

GGTGGTGAAGGTGAGCAAACCAACTACGTCGAGGGCGCGAACATTGCCGGTTTTGTGAAGGTTGCCGATGCG

ATGCTGGCGCAGGGTGTGATTTAAGTTGTAAATGCCTGATGGCCCTAC

GCTTATCAGGCCTACAAATGGGCACAATTCATTGCAGTTACGCTCTAATGTAGGCCGGGCAAGCGCAGCGCC

CCCGCCAAAATTTCAGGCGTTTATGAGTATTTAAGAGCTC

SacI

Fig 7A

Fig 7B 2180783

Mutagenized gdhA for Plant expression with added linker restriction sites (corn)

1

Pst I SalI XbaI EcoRI

ctgcaggtcgacTCTAGAACAATGGATCAGACATATTCTCTGGAGTCATTCCTCAACCATCTCGAAAAG

CGCGACCCGAATCAAACCGAGTTGGCGCAAGCCGTTCGTGAAGTAATCACCACACTCTGGCCTTTTCTT

GAACAAAATCCAAAATATCGCCAGATGTCATTACTGGAGGCTCTCGTTGAA

CCGGAGCGCGTGATCCAGTTTCCGTGGTATGGGTTGATGATCGCAACCAGATACAGGTCAACCGTGCAT

GGCGTGTGCAGTTCAGCTCTGCCATCGGCCCGTACAAAGGCGGTATCGCC

TTCCATCCGTCAGTTAACCTTTCCATTCTCAAATTCCTCGGCTTTGAACAAACCTTCAAAAATGCCCTGA

CTACTCTGCCGATGGGCGGTGGTAAAGGCGGCAGCGATTTCGATCCGAAA

GGAAAAAGCGCAAGGTGAAGTGATGCGTTTTTGCCAGGCGCTGATCACTGAACTGTATCGCCACCTGGGCG

CGGATACCGACGTTCCGGCAGGTGATATCGGGGTTGGTGGTCGTCAAGTC

GGCTTTATGGCGGGGATGATGAAAAAGCTCTCCAACAATACCGCCTGCGTCTTCACCGGTAAGGGCCTTT

CATTTGGCGGCAGTCTTATTCGCCCGGAAGCTACCGGCTACGGTCTGGTT

TATTTCACAGAAGCAATGCTAAAACGCCACGGTATGGGTTTTGAAGGATGCGCGTTTCCGTTTCTGGCT

CCGGCAACGTCGCCCAGTACGCTATCGAAAAAGCGATGGAATTTGGTGCT

CGTGTGATCACTGCGTCAGACTCCAGCGGCACTGTAGTTGATGAAAGCGGATTCACGAAAGAGAAACTGGC

ACGTCTTATCGAAATCAAAGCCAGCCGCGATGGTCGAGTGGCAGATTAC

GCCAAAGAATTTGGTCTGGTCTATCTCGAAGGCCAACAGCGCGTGGTCTTCACCGGTTGATATCGCCCTGCCT

TGCGCCACCGCAGAATGAACTGGATGTTGACGCCGCGCATCAGCTTATC

GCTAATGGCGTTAAAGCCGTCGCCGAAGGGGCAAATATGCCGACCACCATCGAAGCGACTGAACTGTTCCAG

CAGGCAGGCGTACTATTTGCACCGGGTAAAGCGGCTAATGCTGGTGGC

GTCGCTACATCGGGCCTCGAAATGGCACAAAACGCTGCGCGCCTGGGCTGGAAAGCCGAGAAAGTTGACGCA

CGTTTGCATCACATCATGCTGGATATCCACCATGCCTGTGTTCAGCAT

GGTGGTGAAGGTGAGCAAACCAACTACGTGCAGGGCGCGAACATTGCCGGTTTTTGTGAAGGTTGCCGATGCG

ATGCTGGCGCAGGGTGTGATTTAAGTTGTAAATGCCTGATGGCGCTAC

GCTTATCAGGCCTACAAATGGGCACAATTCATTGCAGTTACGCTCTAATGTAGGCCGGGCAAGCGCAGCGCC

CCCGGCAAAATTTCAGGCGTTTATGAGTATTTAAGAGCTC

SacI

Fig 8 2180736

```
   EcoRI          SphI
5' aattcgaaccccttgcatg 3'
3'     gcttggggaagc    5'
```

3' EcoRI SphI adapter - between nosT and plasmid for corn transformation

Fig 9

UB-GDH-PAT Construct

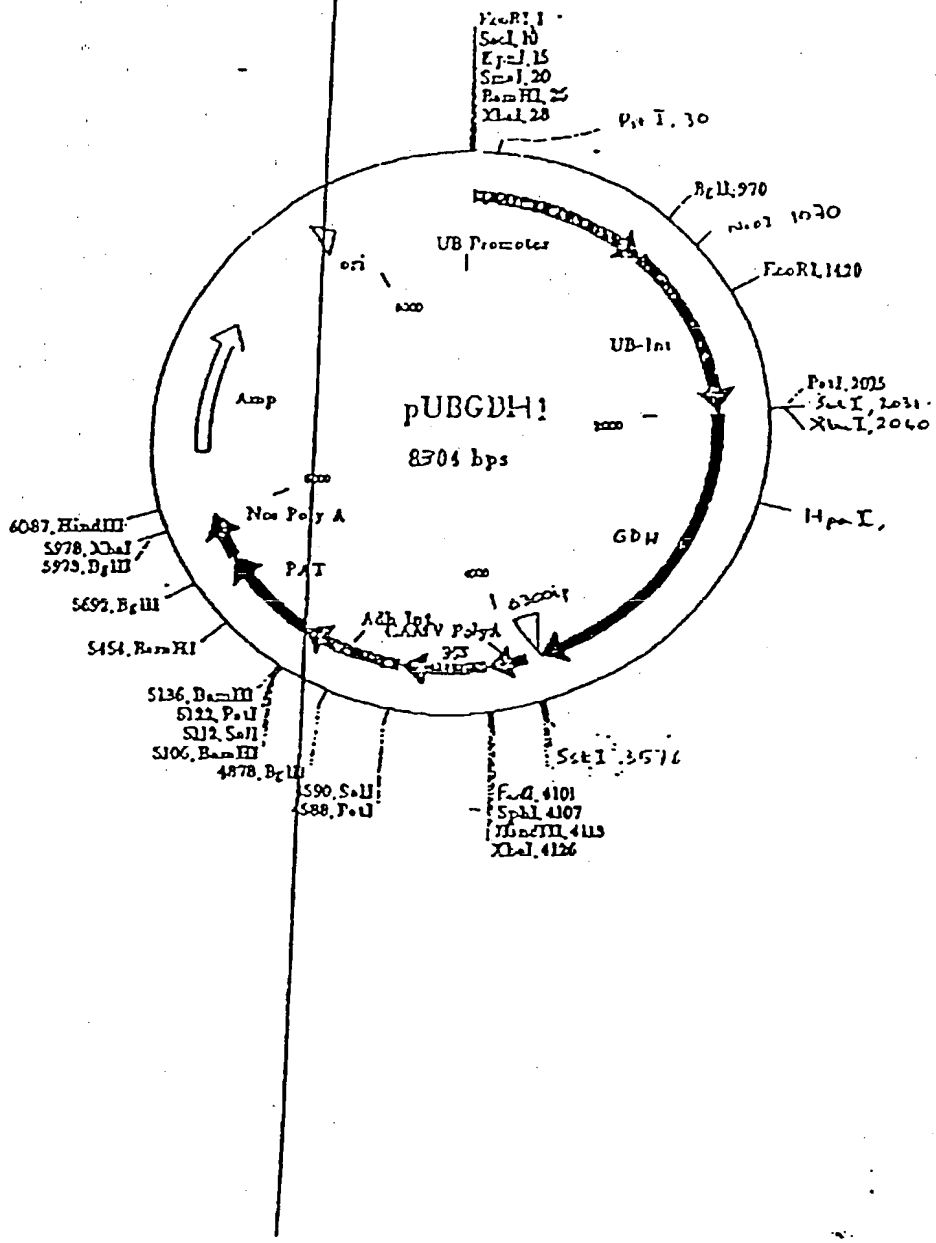
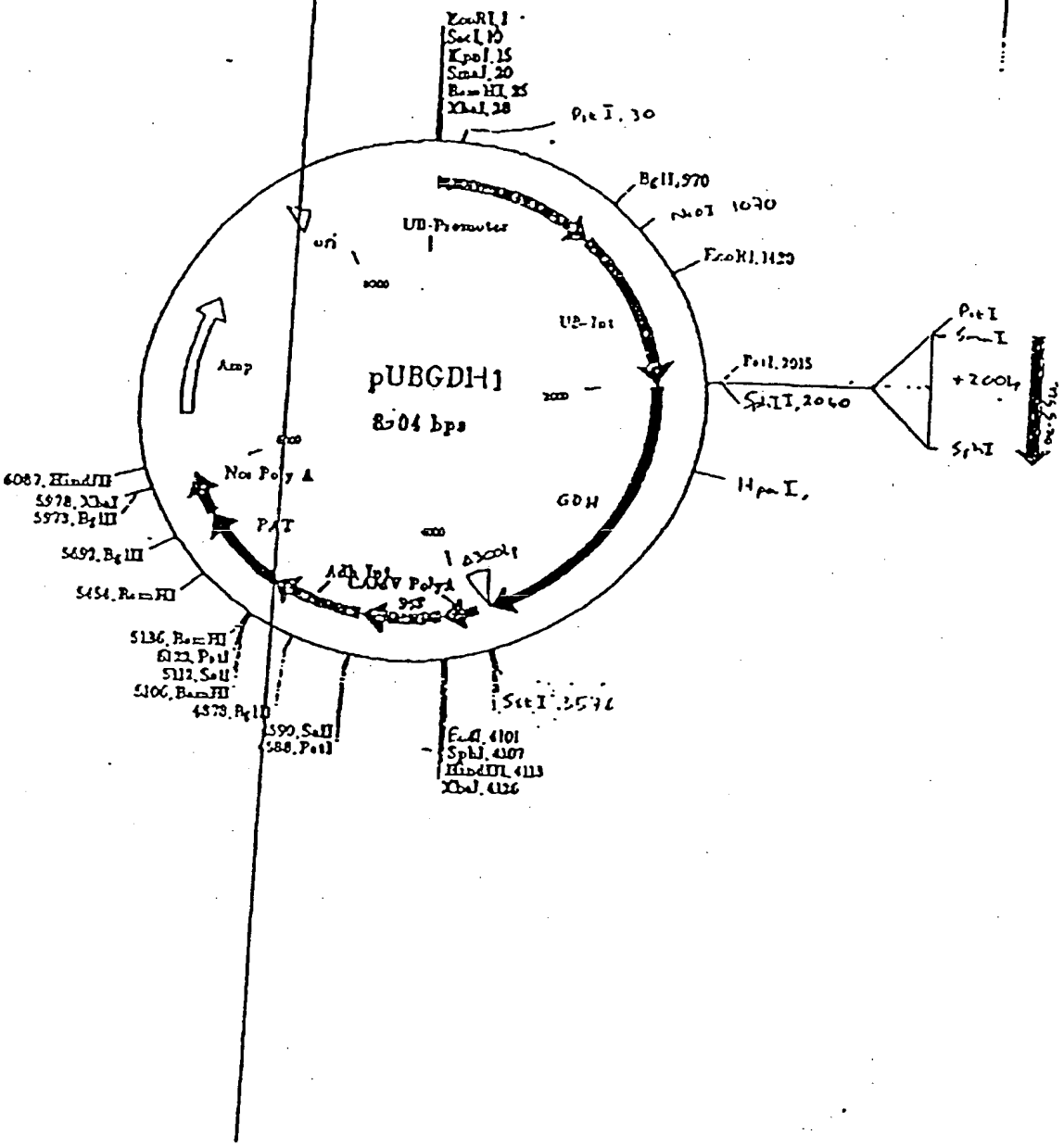


Fig 10

2180786

UB-GDH-PAT Construct



Methylammonium Uptake